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Oestrogenic effects of lucerne in female sheep

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by

Lisa Peers-Adams

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science with Honours.

Oestrogenic effects of lucerne in female sheep

by

Lisa Peers-Adams

Leguminous fodder crops such as lucerne contain oestrogenic substances and their precursors that may interfere with reproduction in sheep. There is no definite information about the actual levels of oestrogenicity of New Zealand lucerne crops and the magnitude of the effect that they have on the reproduction of sheep. There is no information about when a crop may be unsafe for ewes to graze just prior to mating and a simple measurement of sheep is needed to provide an alert so sheep farmers can move sheep off a crop or leave them on knowing that there will be no deleterious effects on subsequent lambing performance. The sheep farm study was an incidental finding from a farmer who noticed udder development in pre-pubertal (25 - 28 weeks of age) lambs (n = 22) grazing lucerne (oestrogenicity ranging from 98.6 – 149.6 µg oestradiol equivalent/kg DM) compared with lambs grazing grass (n = 36) that showed no udder development. Differences in teat length, teat width, mammary development and vulva colour were measured on two occasions – straight after grazing oestrogenic lucerne (12 March) and 35 days later (16 April), 4 weeks after the removal from the lucerne crop. The experimental study was designed to provide a reference calibration with known dosage of an oestrogen. For this 12 Coopworth ewes (42 - 44 weeks of age) were randomly assigned to one of three treatments (n = 4, per treatment): 0, 0,1, 1.0 mg β-oestradiol every 3 days for 40 days. Measurements of teat length, teat width and mammary development was measured every 6 days and, on day 41, the ewes were killed and vulva colour, mammary weight, ovary weight and uterus weight were measured and the number of follicles and corpora lutea were counted. In both studies photographs of the mammary region were taken to provide an alternative measurement technique to use of calipers. Results of the sheep farm study showed lambs grazing oestrogenic lucerne were heavier, had longer teat lengths (2.1 mm difference), with larger teat widths (4.63 mm difference) than sheep grazing grass and mammary development (in 14 out of 25 lambs grazing lucerne) had occurred. There was no change in vulva colour. After removal from lucerne there was a

slight reduction in the oestrogen-related changes and the grass-fed lambs had experienced some mammary/teat development in the intervening period. In the experimental trial, there were dose-related effects on the live weight, teat length, mammary development, uterus weight, ovary weight and mammary weight. There were no changes in teat width, vulva redness or colour saturation, numbers of ovarian follicles and there were no corpora lutea present. Measurements taken from photographs provided reliable information about mammary/teat dimensions. Although the pre-pubertal lambs grazing lucerne were receiving less oestradiol equivalent daily than the older ewes supplied with known amounts of oestradiol (181.2 – 274.9 µg oestradiol equivalent versus 333.3 µg oestradiol per day, respectively), they showed a greater sensitivity to this hormonal stimulus than was exhibited by the pubertal ewes.

Keywords: phyto-oestrogens, oestrogen, sheep, lucerne, reproduction, coumestrol, oestradiol

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Chapter 1

Introduction

Forage plants from the leguminous family are widely used in New Zealand agricultural production systems. The most common forage species used are lucerne (*Medicago sativa* L.), white clover (*Trifolium repens* L.), subterranean clover (*Trifolium subterraneum* L.) and red clover (*Trifolium pratense* L.). These forages potentially contain biologically active oestrogenic compounds called phyto-oestrogens that have a chemical structure similar to naturally occurring oestrogens therefore mimic their activity. The similar chemical structure allows them to act on the oestrogen receptors to induce oestrus and cause morphological changes in the reproductive tract therefore they can reduce the reproductive efficiency of domestic livestock (Adams, 1995; Kurzer and Xu, 1997).

Lucerne is a high quality feed for dryland pastures due to its high water use efficiency and high crude protein content. Coumestrol, a phyto-oestrogenic compound, is present in lucerne and it has the potential to reduce the ovulation rate of ewes that graze it during or prior to mating. The concentration of coumestrol in lucerne is highly variable and highly dependent on a number of environmental factors therefore is not always potent enough to exert an oestrogenic effect (Oldfield *et al*, 1966). The level of oestrogenic compounds in lucerne is considerably less than those reported in subterranean clover therefore the effects on reproduction are not as prominent. These effects include decreased lambing rates, decreased ovarian activity and reduced ovulation rate (Coop, 1977; Newsome and Kitts, 1977; Scales *et al.,* 1977). Lucerne does contain other phyto-oestrogens, such as formononetin which is considered the most prominent isoflavone that influences reproduction in domestic livestock, however, these only accumulate in small quantities in lucerne compared to coumestrol (Adams, 1995; Nwannenna *et al*., 1995).

Phyto-oestrogens have a similar structure to oestradiol and behave like endogenous oestrogens. In their active form they bind with oestrogen receptors. This binding causes detectable changes in the reproductive tract of female sheep. Phyto-oestrogens can effect uterine events and induce increased udder and vulva development (Oldfield *et al.,* 1966; Newsome and Kitts, 1980). Problems can be measured by reduced lambing rates and changes in mammary gland, vulva, and teat parameters.

The aim of this study was to measure the stimulatory effects of oestrogenic lucerne on the mammary glands and teats of pre-pubertal sheep in New Zealand and to relate the oestrogenic effects recorded on-farm to those produced by a precise dose administration of oestradiol to sheep. It also aimed to

demonstrate the utility of photographic procedure to replace direct measurements of mammary and teat development and oestrogen-induced colour changes in external genitalia.

Chapter 2

Literature Review

2.1 Introduction

Phyto-oestrogens mimic endogenous oestrogens and bind with oestrogen receptors exerting oestrogenic effects on the central nervous systems causing changes in the reproduction tract of sheep (Kurzer and Xu, 1997). Over 300 plants have high enough levels of oestrogenic activity through phyto-oestrogen concentration to be able to initiate oestrous behaviour in animals (Kurzer and Xu, 1997). Phyto-oestrogens and their effects has been widely studied in laboratory animals, humans, sheep and cattle.

This literature review covers oestrogens and their role in the development and the regulation of reproduction systems and the chemical structure and metabolism of phyto-oestrogens and the effect that they have in sheep with particular reference to their potential to reduce reproductive performance on farms. In addition, the morphological changes associated with increased oestrogen and phyto-oestrogen consumption is described.

2.2 Oestrogens

Oestrogens are a group of steroid hormones that are involved in the regulation of reproduction, particularly in females. They are comprised of four carbon rings, one of these (A-ring) being aromatic, and have a total of 18-carbon atoms. Naturally occurring oestrogens are steroid based, as are other sex hormones involved in reproduction such as the progestogens (21 carbons) and the androgens (19 carbons). Steroid based hormones are derived from cholesterol, a 27-carbon molecule. Cholesterol binds to lipoprotein receptors and is taken up by steroidogenic cells where it is stored and transported to the sites of steroid synthesis. During steroid synthesis, the different steroid-based hormones are formed through a reduction in carbon numbers (Gruber *et al.,* 2002). The three major naturally occurring oestrogens are 17-β oestradiol, oestrone and oestriol. They are chemically similar to each other yet have different roles in the body and are present at differing concentrations throughout the body. Oestrone predominates in domestic livestock during pregnancy while oestradiol is in abundance in the non-pregnant animal (Reece, 1991). The primary sources of oestradiol are the theca and granulosa cells of the ovaries, however other tissues such as adipose tissue have the ability to synthesise oestradiol therefore contributing to its overall concentration in the body (Nelson and Bulun, 2001; Gruber *et al.,* 2002).

2.2.1 Role of oestrogens in ruminants

Oestrogens play a role in many physiological processes. In the brain they have been shown to regulate the activity of many neurotransmitters and neuropeptides systems by altering the concentration of their receptors or acting at the synthesis/release level (Balthazart *et al.,* 2009). However, their principle function is the development and regulation of the reproductive system. They are involved in cellular proliferation and growth of reproductive organs. This includes: stimulation of the endometrial gland growth, stimulation of mammary gland duct growth and increasing secretory activity of uterine ducts (Reece, 1991). They also have involvement in feedback control of gonadotropin secretion, stimulation of follicular growth and maturation, initiation of sexual receptivity and some involvement in creating a favourable uterine environment for fertilised egg cells to develop and helping the uterus prepare for parturition (Balthazart *et al.,* 2009).

For female conception to be successful, ovulation needs to occur. An increase in the oestradiol concentration during ovulation triggers the gonadotropin releasing hormone (GnRH) surge, which generates the preovulatory surge of luteinizing hormone (LH). This is a positive feedback mechanism of oestradiol that works by binding oestradiol to oestradiol receptors (ERα) located in specific hypothalamic nuclei, having a stimulative effect in the surge process resulting in the observed surges of GnRH and LH. This surge mechanism involving oestradiol is demonstrated in females of most species (Gordon, 1997; Clarkson and Herbison, 2009). The number of oestradiol receptors in the anterior pituitary gland increases the sensitivity of gonadotrophs to GnRH (Nett *et al*., 1984).

2.3 Phyto-oestrogens

Phyto-oestrogens are diphenolic, oestrogenic, plant-derived compounds that have a similar chemical structure to oestrogens and can bind with oestrogen receptors causing an effect. Although they mimic the behaviour of oestradiol, their effects are not always interchangeable (Adams, 1995). Phyto-oestrogens can be chemically described as being composed of ‘a planar ring system that includes a p-hydroxyl-substituted aromatic ring, approximately 12 A° away from a second planar hydroxyl group’ (Turner *et al*., 2007).

Phyto-oestrogens can be classified into three main chemical classes - coumestans, isoflavones and lignans. Each phyto-oestrogens has a slightly different chemical structure although the structures of all the classes are similar to natural and synthetic oestrogens and anti-oestrogens (Kurzer and Xu, 1997). These slight differences result in the biological activity of the different classes having variation (Turner *et al*., 2007). Phyto-oestrogens exert oestrogenic effects on the central nervous system, induce oestrus and stimulate growth of genital tracts in females (Kurzer and Xu, 1997). These effects

are exerted through a single nuclear receptor known as oestrogen receptors and two forms exist – oestrogen receptor α and oestrogen receptor β (Turner *et al.*, 2007). These receptors are located in reproduction-associated tissues as well as in the heart, brain, liver and bones. Oestrogen receptor α is primarily expressed in the gonadal tissues and also in low concentrations in other tissues such as the kidney, the brain and adipose tissue, whereas oestrogen receptor β is primarily expressed in non-gonadal tissue such as colon, bone marrow and the brain (Cui *et al.*, 2013). The affinity of phyto-oestrogens to bind to the oestrogen receptors varies depending on the substrate. Some substrates have equal affinities for both receptors, some have an increased affinity for a particular oestrogen receptor and some selectively bind to a single oestrogen receptor. For example, isoflavones are capable of binding to both of the oestrogen receptors, however genistein, an isoflavone, has a 7 – 30 times greater binding affinity for oestrogen receptor β. This is considered relatively selective binding (Turner *et al.*, 2007).

2.3.1 Classes of phyto-oestrogens

A single plant will often contain more than one class of phyto-oestrogen. Out of the three main classes, isoflavones and lignans have been identified as being the most prevalent (Kurzer and Xu, 1997; Murkies *et al.*, 1998). Isoflavones are a type of isoflavanoid, which are naturally occurring, biologically active compounds. They are almost exclusively produced in the Fabaceae family, which includes beans, legumes and peas. Formononetin, genistein and daidzein are all examples of isoflavones and all exert different levels of oestrogenic activity, which depends on the plant species that they are present in. Formononetin itself is not an oestrogenic substrate but in the rumen it is metabolised mainly to equol, which is oestrogenic. It is present in red clover and believed to be the main cause of reproductive problems such as permanent infertility in sheep that have had prolonged exposure to red clover (Cox and Braden, 1974; Kurzer and Xu, 1997; Dixon, 2004).

Coumestrol is the main phyto-oestrogen of the coumestan classification. It can be found in oestrogenic forages such as lucerne, however, coumestans levels are usually low and do not exert an effect. Coumestrol levels in lucerne can rise due to an array of factors and when they are present in concentrations higher than 20-50 mg/kg of DM then it exerts an effect on the reproductive system such as increasing reproductive organ development in sheep. It has the greatest activity of any of the phyto-oestrogen compounds studied and its potency is 1/1000th that of oestradiol 17-β (Wong *et al.*, 1971; Newsome and Kitts, 1980; Adams, 1986; Adams 1995).

Lignans commonly occur throughout plants due to their role in plant defence. They are commonly found in rye and flaxseeds and in the outer layers of cereals and grains (Dixon, 2004). Only certain

types of plant lignans are of importance in terms of exerting an oestrogenic effects and these are the ones that are converted to enterolactone and enterodiol by the intestinal microflora. Examples of lignans converted to enterolactone and enterodiol are secoisolariciresinol and matairesinol (Wang *et al.,* 2002).

2.3.2 Metabolism of phyto-oestrogens

The metabolic fate of phyto-oestrogens influences the oestrogenic effect that it exerts due to metabolism either increasing or decreasing oestrogenic activity (Adams, 1995; Lundh, 1995). Due to differences in metabolism between ruminants and monogastrics, they break down and metabolise phyto-oestrogens differently. In ruminants, metabolism of phyto-oestrogens is directly affected by the ruminal microflora as they readily hydrolyse the phyto-oestrogens (Cheeke, 1998). The micro-organisms in the rumen can take between 6 – 10 days to adapt to substrates such as genistein and biochanin A, resulting in them exerting their oestrogenic effects for the first couple of days following the introduction of an oestrogenic pasture but not having any long-term effects (Adams, 1995). Metabolism of phyto-oestrogens depends on the class of phyto-oestrogen due to the slight differences in the chemical structure (Kurzer and Xu, 1997).

Coumestrol is absorbed into an animal in its active form and can be detected in the plasma as soon as 1 hour following ingestion (Kelly and Lindsay, 1978). Demethylation can increase the oestrogenicity by increasing the substrate affinity for oestrogen receptors. For example, 4'methoxy-coumestrol has greater oestrogenicity following demethylation, which allows the hydroxyl group to bind to the oestrogen receptor (Adams, 1995). Free coumestrol, which is oestrogenically active, accounts for 20 - 40% of the circulating coumestrol (Kelly and Lindsay, 1978).

The length of time that phyto-oestrogens stay in the rumen, affects their metabolism. The retention time of isoflavones in the rumen is the main contributor to their degradation and detoxification (Livingstone, 1978; Lundh *et al.,* 1990). Rumen microorganisms break down isoflavones such as biochanin A and genistein into their non-oestrogenic metabolites (Cox and Braden, 1974). Formononetin is the most important isoflavone in terms of long-term oestrogenic effects in ruminants consuming subterranean and red clover (Lundh *et al.,* 1990; Adams, 1995). Formononetin has low oestrogenicity however it is demethylated to daidzein and further metabolised via hydrogenation to equol, which is a highly oestrogenic compound and is rapidly absorbed through the ruminal wall and gastro-intestinal tract. Equol is thought to be the major oestrogenic compound that initiates oestrogenic effects in sheep (Nilsson *et al*., 1967; Shutt and Braden, 1968; Lundh *et al*., 1990; Adams, 1995).

The metabolism of phyto-oestrogens is similar in cattle however there are minor differences (Adams, 1995). Cattle appear to be less sensitive and less affected by phyto-oestrogens than sheep (Lundh, 1995). At the same relative plasma concentration, isoflavones have been shown to have less of an effect on cattle than sheep (Adams, 1995). Differences between the metabolism of phyto-oestrogens of sheep and cattle have been contributed to their differing concentrations of oestrogen receptors and their ability to competitively bind. Sheep uterine concentration of oestrogen receptors is two to four times higher than in cattle. Sheep are also more efficient at conjugating phyto-oestrogens than cattle – conjugating is an important mechanism in animals to be able to detoxify feed substances including plant oestrogens (Lundh, 1995).

2.4 Oestrogenic pastures

Oestrogenic plants are common throughout the plant kingdom, more so in legumes. Legume species are widely used in agriculture production systems due to their high protein content and their ability to fix nitrogen. Isoflavones are most commonly found in pastures; namely subterranean clover, red clover and soybean, and the most common sources of coumestans are lucerne, white clover and the medics. The dietary concentration of phyto-oestrogens needed to exert an oestrous effect is relatively similar across species (Adams, 1996).

The phyto-oestrogen content of forages is highly variable. Genetic variation is responsible for a significant portion of phyto-oestrogen variation in plants. This is evident with isoflavone variation in clover varying with different subterranean clover strains (Millington *et al.,* 1964; Nicollier and Thompson, 1982). The accumulation of phyto-oestrogens and the potency of forages also depends on the season, environmental factors, cultivar, age and stresses such as disease and fungi (Oldfield *et al*., 1966). The plant part also differs in phyto-oestrogen concentration and in lucerne a variation has been observed between the stem, leaf and flower of the plant. The difference in phyto-oestrogen content of the plant part and the proportions of plant components in a stand impact the phyto-oestrogen content of harvested material (Seguin *et al*., 2004). The variability in phyto-oestrogen concentrations makes it difficult to get an accurate measurement of the oestrogenicity of a crop.

2.5 Effects of phyto-oestrogens in ruminants

The effect that phyto-oestrogens exert in terms of oestrous behaviour has been widely studied in ruminants, mainly cattle and sheep. The most damaging effect that phyto-oestrogens have on ruminants is the potential to suppress fertility through a reduction in the ovulation rate. Phyto-oestrogens can cause temporary and permanent effects on the fertility of an animals

2.5.1 Clover disease

'Clover disease' refers to the long term infertility in sheep after grazing oestrogenic clover pastures, subterranean and red, for a prolonged period. 'Clover disease' is characterised by uterine prolapse, difficult lambing and permanent infertility of ewes. The ability of ewes to conceive is reduced therefore an increase is observed in the number of 'dry' ewes in a flock (Adams, 1986). Subterranean clover and red clover are both oestrogenic pastures typically high in isoflavone phyto-oestrogens such as genistein, daidzein and their precursor's biochanin A and formononetin. Formononetin level was correlated with oestrogenicity in clover pastures and although not highly oestrogenic itself, in the rumen it is metabolised into equol, which is highly oestrogenic (Kurzer and Xu, 1997). Infertility caused by 'clover disease' is no longer common due to a larger proportion of grass being used in clover pastures and through the use of low-formononetin subterranean clover varieties (Adams, 1995).

2.5.2 Temporary infertility

The pasture that a sheep is mated on can temporarily impact an animal's fertility. Consumption of phyto-oestrogens during mating can contribute to temporary infertility characterised by a reduction in lambing rates, multiple births, conception from first service, conception rates, irregular oestrus and an increase in the amount of spontaneous abortions, death of newborns, difficulty birthing and uterine prolapse observed in a flock (Newsome and Kitts, 1980; Adams, 1995; Vetter, 1995; Valderrbona *et al*., 1988). This infertility is temporary and usually resolves itself several weeks to a month following removal of animals from an oestrogenic pasture to a non-oestrogenic pasture (Adams, 1995). It is difficult to attribute a temporary drop in ovulation rate to phyto-oestrogens due to the ovulation rate varying from live weight, stage of breeding season and nutritive value of the pasture (Adams, 1995).

2.5.3 Permanent infertility

An increase in phyto-oestrogen concentration in plants has shown to be correlated with a decrease in fertility of ewes grazing oestrogenic pastures (Livingstone, 1978). Prolonged exposure to oestrogenic pastures can cause permanent infertility caused by a reduction in the ability of ewes to conceive due to the cervix not permitting the normal transport of the spermatozoa (Lightfoot *et al*., 1974). The structure of the cervix also changes and starts to resemble a uterus in both function and appearance. Histologically, permanent infertility is easy to detect through the changes in the cellular composition of the cervix (Adams, 1986).

2.6 Morphological changes associated with oestrogens and phyto-oestrogens

The consumption of phyto-oestrogens through oestrogenic pastures results in morphological changes in an animal. The same changes are observed when animals are administered oestrogens, such as oestradiol 17-β, either through oestrogen implants or injections. The morphological changes associated with phyto-oestrogen consumption or oestrogen administration include teat size parameters, udder development, vulva colour change, uterine size and cervix histology.

2.6.1 Teat size

Elongation of the teat length due to oestrogen consumption has been observed in sheep and castrated weather lambs and this response is well characterised throughout literature. Galbraith *et al.* (1997) treated 6-month old ram lambs with oestradiol 17-β and observed an increase in the teat length of the lambs compared to controls. Control lambs had teat length of 8.17 mm while the oestrogen treated ram lambs experienced an increase to 12.1 mm (Galbraith *et al.,* 1997). An increase in teat length has also been observed for wethers grazing oestrogenic pastures. Consumption of red clover has been shown to cause an increase in teat length of 2.5 mm compared with ryegrass, which showed a decrease in teat length of 0.35 mm (Braden *et al.*, 1971). This effect has been observed for a long time, with a mob of wethers experiencing teat length enlargement (up to 2-4 cm) and teat base enlargement (up to 2 cm) after consuming a diet largely comprised of red clover in 1954. These wethers also experienced mammary secretions (Cunningham and Hogan, 1954). Nwannenna *et al.* (1995) and Oldfield *et al.* (1966) both observed an increase in teat size in female sheep. Nwannenna *et al.* (1995) measured the teat length of ewes fed red clover and all of them significantly increased in size, which later decreased back to normal size 25 days after removal from red clover. The study also looked at the effects of oestradiol 17-β implants and the response was much more abrupt with noticeable changes occurring by day 4 and sheep taking 19 and 26 days to return to pre-treatment teat length (90 µg/day and 45 µg/day release, respectively) (Nwannenna *et al.,* 1995). An increase in teat length can be a simple measure of the oestrogenicity of plants, however, this is an imprecise method (Adams, 1995).

2.6.2 Mammary gland size

Grazing oestrogenic pastures can lead to an increase in the mammary development of sheep. Non-pregnant four-year old ewes grazing oestrogenic subterranean clover had increased mammary gland development and experienced milky secretions from the mammary gland (Adams, 1977). Valderrabano *et al.* (1988) compared 3-month-old lambs grazing on lucerne, an oestrogenic pasture,

with those grazing on Italian ryegrass, a typically non-oestrogenic pasture. The higher phyto-oestrogen content of lucerne led to a significant increase in the development of mammary tissue; 193.0 g for sheep grazed on lucerne compared with 129.7 g for sheep grazed on Italian ryegrass. The increase in mammary gland development can be attributed to an increase in the amount of glandular tissue development and a decrease in the development of connective tissue. Consumption of oestrogenic feeds also caused an increase in size of the reproductive tract; 181.22 g for sheep grazed on lucerne compared with 165.00 g for sheep grazed on Italian ryegrass (Valderrabano *et al*., 1988).

2.6.3 Vulva colour change

Nwannenna *et al*. (1995) assessed the colour of the vulva in ovariectomized ewes on two separate occasions by visual appraisal. The first experiment occurred over short days and sheep were given red clover silage corresponding to 6.1g of phyto-oestrogens. A gradual change from pale to pink and then to red was observed with increasing oedema. In the second experiment the ewes were given oestradiol 17-β implants (45 or 90µg), once again during short days. The vulva colour changed abruptly from pale pink to red with obvious oedema. The change in vulva colour was thought to be due to increased blood flow that accompanies hyperplasia and hypertrophic enlargement of the reproductive organs when exposed to oestrogens (Nwannenna *et al.,* 1995). Measuring vulva colour using this method, visual appraisal, can be biased and it may be more accurate to assess vulva colour using an objective technique such as measuring the colour saturation of the vulva. Colour saturation changes of the vulva associated with oestrogen concentration has been undertaken in dogs. It has been suggested that elevated oestrogen levels cause proliferation of the cellular structure and oedema of the mucosal folds, hiding the underlying blood capillaries. In theory, by the end of the fertile period colour saturation would increase due to decreased oedema and a reduction in cellular layers (Moxon *et al.,* 2012). Both studies had a small sample size therefore whether there is a colour change of the vulva associated with oestrogen concentrations has not been confirmed.

2.6.4 Uterine size

Oestrogens have been shown to be responsible for proliferation of the cells in the uterus resulting in uterine gland development. Oestradiol and phyto-oestrogen dosing of lambs results in increased uterine growth, which was real growth rather than water accumulation (Newsome and Kitts, 1980). In an experiment, control sheep were compared to those dosed with differing oestradiol concentrations (2.5 mg/day, 10 mg/day and 50 mg/day) and coumestrol (132 µg/day). All sheep dosed with oestradiol and coumestrol experienced an increase in uterine weight; sheep dosed with 10 mg oestradiol/day experienced the maximum uterine weight response. Due to this, it was

concluded that the oestradiol dose of 50 mg/day was too high and the response when dosed at 2.5 mg/day was more likely to be physiological (Newsome and Kitts, 1980).

Johnson *et al*. (1997) also noticed a dramatic increase in the uterine size following administration of ewes with oestradiol- 17β. Ovariectomized ewes treated with oestradiol had an average uterine weight of 64.8 g compared with those who received no treatment whose mean uterine weight was 27.0 g (Johnson *et al*., 1997). Looking at the course response of the uterus to oestrogen implants (50 mg E_2, 2 implants per ewe) showed that the fresh weight of the uterus doubled in size in the first 24 hours following oestradiol administration – increasing from 20.6 to 46.8 g. Between 0 and 48 hours, the uterus weight underwent a 3.3-fold increase – from 20.6 g to 68.6 g. Uterine growth response was primarily due to tissue growth rather than an increase in the dry weight to wet weight ratio. Tissue growth was contributed equally to hyperplasia (increase in cell number) and hypertrophy (increase in cell size) in the first 24 hours following treatment (Reynolds *et al*., 1998).

2.6.5 Ovary weight

Consumption of phyto-oestrogens and oestrogens has shown to decrease ovarian weight of pre-pubertal lambs. In a study described by Newsome and Kitt (1980), pre-pubertal lambs were dosed with either an oestrogen or coumestrol or were controls. The ovarian weights of the lambs given oestradiol and coumestrol were lower than those of the controls. The reduction in ovarian weight could result in reduced or altered ovarian function (Newsome and Kitt, 1980). Valderrabano *et al*. (1988) also noticed a suppression in ovarian size in ewe lambs grazing oestrogenic lucerne compared to those grazing Italian ryegrass; 0.53g and 0.67g, respectively. Likewise, Smith *et al*. (1990) observed the ovary size of sheep after administration of zearalenone, a phyto-oestrogen. Ewes (5-6 years old) were either administered 0.0, 1.5, 3.0, 6.0, 12.0 or 24.0 mg zearalenone per day for 10 days. The trend showed that the higher the dose of zaeralonone, the smaller in weight of the ovaries. The only exception to this was at the dose of 1.5 mg zaeralonone per day, where a slight increase in ovarian weight was observed compared with the control ewes (Smith *et al.,* 1990).

2.7 Summary

Phyto-oestrogens are found mainly in leguminous pastures such as lucerne and clover and can have deleterious effects on animal reproduction, particularly by causing a reduction in ovulation rate which manifests as fewer multiple births. It is recommended that farmers remove ewes from Lucerne-dominant pastures well prior to mating to ensure that the reproduction rate of breeding ewes is not compromised. However, removing sheep from lucerne pastures results in the inability to utilise high quality feed that can increase the body condition of ewes prior to mating. Oestrogen and

oestrogenic pastures alike cause morphological changes that can be quantified. These changes include a change in teat size, vulva colour, uterine size, ovary weight and mammary gland development. Teat size, uterine size and mammary gland development all increase under the influence of oestrogens while vulva colour becomes increasingly darker. Ovarian weight decreases with increasing oestrogen concentrations and ovarian activity also decreases. These changes could potentially be used to detect when sheep have consumed high levels of phyto-oestrogens and management decisions could be made about timing of removal of sheep from oestrogenic crops to prevent any impairment of their reproductive performance.

Chapter 3

Materials and Methods

3.1 Sheep farm study

Apparent udder development was observed in ewe lambs grazing lucerne on a commercial farm, 'Creedmore' located 17 km southwest of Oamaru, Canterbury, New Zealand. Sheep were cross-bred ewe lambs of Texel-East Friesian-Coopworth ancestry, born in spring 2014 (25 – 28 weeks of age) and at weaning (27 November) they were allocated to either a lucerne crop (Lucerne) or remained on rye-grass/white clover pasture (Grass). This allocation was based on their live weight; the lighter lambs being those allocated to the lucerne pasture.

On 12 March measurements of the sheep were taken (as described below) for 25 of the Lucerne lambs and 10 of the Grass lambs. Lucerne lambs were returned to the rye-grass/white clover pasture with the Grass lambs on 20 March and another set of measurements was recorded on 16 April prior to the introduction of rams. Live weight of all animals was recorded each month and mating activity was recorded daily by use of crayon-harnessed rams. On 24 June the number of fetuses present in each ewe was determined by trans abdominal ultrasonography carried out by a commercial operator.

Post hoc analysis (yeast cell bioassay) of the lucerne crop on 12 March showed it to have an oestrogenicity of 151.4 µg oestradiol equivalent /kg DM with a coumestrol level of 8.25 mg/kg DM and the crop Lucerne sheep moved onto the same day had an oestrogenicity of 99.8 µg oestradiol equivalent /kg DM with a coumestrol level of 8.32 µg /kg DM.

3.1.1 Measurements

Live weight

Non-fasted live weight was recorded using electronic scales.

Teat and mammary gland size

Measurements of teat and mammary dimensions were taken whilst each lamb was held manually in a semi prone position.

Teat length and teat width at the base were measured with digital calipers. If udder development was visually prominent, its diameter was measured with calipers. Each udder was photographed with

a ruler present in the view field so that a separate measure of teat length and width was recorded from the photographic images.

Vulva colour

A photograph was taken of each lamb's vulva to enable the colour of the internal mucosa to be evaluated. The images were saved as 2592 X 3888 pixels JPEG images with a horizontal and vertical resolution of 72 dpi and a bit depth of 8. Colour readings were obtained using ColorPix (http://www.colorschemer.com/ colorpix_info.php). The colour saturation was measured five times for each image and the mean value was recorded, avoiding areas where the light source affected the image.

3.2 Experimental study

Twelve non-pregnant female Coopworth sheep, aged between 42 and 44 weeks of age (mean live weight = 42.75 ± 5.58 kg) were randomly allocated into three groups (n = 4) that were balanced for live weight. They were located on the Research Farm, Lincoln University, Canterbury, New Zealand (43°38'54" S 172°27'24" E, at an altitude of 9m) and were grazed on pasture consisting predominantly of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) with water available ***ad libitum***. For a period of 40 days, commencing on 26 July, the ewes were treated every 3 days with either 0, 0.1 or 1.0 mg of oestradiol, depending on group, so that they received 13 treatments during the experimental period. Throughout the study live weight and other measurements (as described below) were taken at regular intervals after the ewes had been mustered from their paddock and moved into a nearby yard. At completion of the study (Day 41) the ewes were killed, using captive bolt pistol for stunning followed by exsanguination, and reproductive tracts and mammary glands were removed for weighing. All animal procedures were approved by the Lincoln University Animal Ethics Committee.

3.2.1 Oestradiol treatment

Oestradiol (β-estradiol, Sigma St Louis, MO. Cat E8875) was dissolved in a small volume of ethyl alcohol and the solution was added to sunflower oil (Sunfield Oil, Auckland, New Zealand) that had been rendered sterile by filtering it using a 0.22µm filter (Micron Separations Inc., Westborough, MA, USA). The oil, either without oestradiol or containing oestradiol, was stored in separate 15 ml conical polypropylene centrifuge tubes (Corning, New York, USA) so that 0.5 ml of the solution delivered 0, 0.1 or 1.0 mg oestradiol (depending on sheep group) when it was administered by intramuscular injection into the neck using a 1 ml syringe and 18-gauge needle (Becton Dickinson and Company,

Franklin Lakes, NJ, USA). This was done on the first and every subsequent third day whilst the animals were held by manual restraint in a holding yard.

3.2.2 Measurements

Live weight

Non-fasted live weight was recorded using electronic scales on Days 1, 21 and 40.

Teat and mammary gland size

Teat and mammary gland dimensions were measured on Day 1 and every 6 days thereafter (i.e. 7 times) whilst each ewe was held manually by an assistant in a semi prone position.

Teat length and teat width at the base were measured with digital calipers. If udder development was visually prominent, its diameter was measured with calipers and its circumference determined with a measuring tape. Each udder was photographed with a ruler present in the view field so that a separate measure of teat length and width was recorded from the photographic images.

Blood sampling

Venous blood samples (10 mL) were obtained on Days 1 and 41 by venepuncture from an external jugular vein whilst the sheep were manually restrained using evacuated plastic tubes containing sodium ethylene diamine tetra acetic acid (K2E, B D Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ, USA) as anticoagulant and a 0.9 x 25 mm needle (PrecisionGlide™, Becton Dickinson and Company). Immediately on withdrawal of the sample, each tube was gently inverted a few times to ensure dispersal of the anticoagulant. Soon after collection blood samples were centrifuged at 1000 *g* in a bench centrifuge for 15 minutes and plasma was collected by aspiration and placed in 12x75mm polycarbonate tubes to be stored frozen. (Note: the plasma sample obtained on day 41 was collected approximately 96 hours after the preceding dose of oestradiol had been administered to each ewe.)

Reproductive tract

At slaughter the vulva and about 2 cm of vagina was cut from each reproductive tract with scissors. A separate photograph was taken for each group of ewes' vulvas to enable the colour of the internal mucosa to be evaluated. Photographs were taken with a FAMACHA © chart in the field of view to use as a reference value. The images were saved as 2448 X 3264 pixels JPEG images with a horizontal and vertical resolution of 72 dpi and a bit depth of 8. Colour readings were obtained using ColorPix (http://www.colorschemer.com/colorpix_info.php). The colour saturation and colour redness were both measured five times for each image and the mean value was recorded avoiding areas where the

light source affected the image. The mean values were corrected for variation in light by using the FAMACHA © chart reference value.

The uterus was separated from the vagina by cutting through the tract at the level of the cervix os. The ovaries were removed by dissection and extraneous ligamental tissue removed from the uterus by blunt dissection. Wet weight of the uterus and each individual ovary was measured using electronic scales. The ovaries were inspected visually for presence of corpora lutea and the number of large follicles (between 5 and 7 mm, and above 8 mm) was recorded. The data for each ovary were pooled to give a single total value for each ewe.

Mammary gland

The mammary gland, as a single organ, was dissected free from the skin and underlying peritoneum and its wet weight was measured using electronic scales.

3.3 Statistical analyses

Results were analysed using Excel and Minitab 17.

Minitab was used to analyse the significant ($\alpha = 0.05$) effects on the variables from oestradiol treatment using general analysis of variance model. It was used for live weight, teat size, vulva colour and reproductive tract measurements (uterus weight, mammary gland weight and ovary weight). For comparisons of the means, all standard error of differences (SED) have been calculated at the $P < 0.05$ level.

Minitab was used to analyse the differences over the period of measurements using a Paired *t*-test. Mammary development and teat size were analysed by this approach to determine if there was any change over time. A Paired t-test was to compare measurements taken using the calipers with those determined from the photographs.

Excel was used for regression modelling to see if there was a live weight effect on mammary gland weight, uterus weight, ovary weight and teat length and width. Regression modelling was also done to compare caliper measurements with measurements taken with a photograph and a ruler in present view.

Ovarian follicle numbers were analysed by use of a Chi-square test.

Chapter 4

Results

4.1 Sheep farm study

4.1.1 Live weight

Live weights of the sheep recorded on two occasions about 4 weeks apart are provided in Table 4.1. Grass lambs (n = 22) were heavier ($P < 0.01$) than Lucerne lambs (n = 36) on each occasion. The Lucerne lambs had a higher ($P < 0.001$) gain in live weight during that period (Table 4.1).

	Live weight (kg) March	Live weight (kg) 16 April	Live-weight gain (kg)
Grass n = 22	53.2 ± 0.77	58.3 ± 2.02	4.1 ± 0.63
Lucerne n = 36	45.4 ± 0.87	52.4 ± 2.34	7.4 ± 0.74

Table 4.1 Mean (± s.e.m.) live weight of ewe lambs fed on ryegrass/clover pasture (Grass) or lucerne (Lucerne) recorded on 2 occasions in a period of 35 days during late summer 2015 and their mean (± s.e.m.) live weight gain during this period.

4.1.2 Mammary development

Some (14 out of 25) of the ewe lambs grazing Lucerne had visible evidence of mammary gland development on 12 March (see Appendix A). For these lambs, mean mammary gland diameter was 65.8 ± 7.52 mm (n = 14) on 12 March and was unchanged ($P = 0.133$) 35 days later (mean diameter 61.8 ± 6.40 mm, n = 11) on 16 April. There was no visible evidence of mammary development in the Grass lambs on either occasion.

4.1.3 Teat size

There was a diet-related effect on teat lengths recorded on 12 March (Figure 4.1) with longer ($P < 0.05$) teats on Lucerne lambs (mean 22.7 ± 1.20 mm, n = 25) than the Grass lambs (mean 20.6 ± 1.45 mm, n = 10). However, on 16 April, teat lengths had decreased in Lucerne lambs (by 2.28 ± 1.28 mm, to a mean length of 20.4 ± 1.31 mm, n = 22) whereas there had been an increase in teat length of

Grass lambs (by 1.31 ± 1.81 mm to a mean length of 21.9 ± 1.52 mm, n = 10) so that, at this date, there was no difference in teat length between the two groups of sheep (*P* = 0.16).

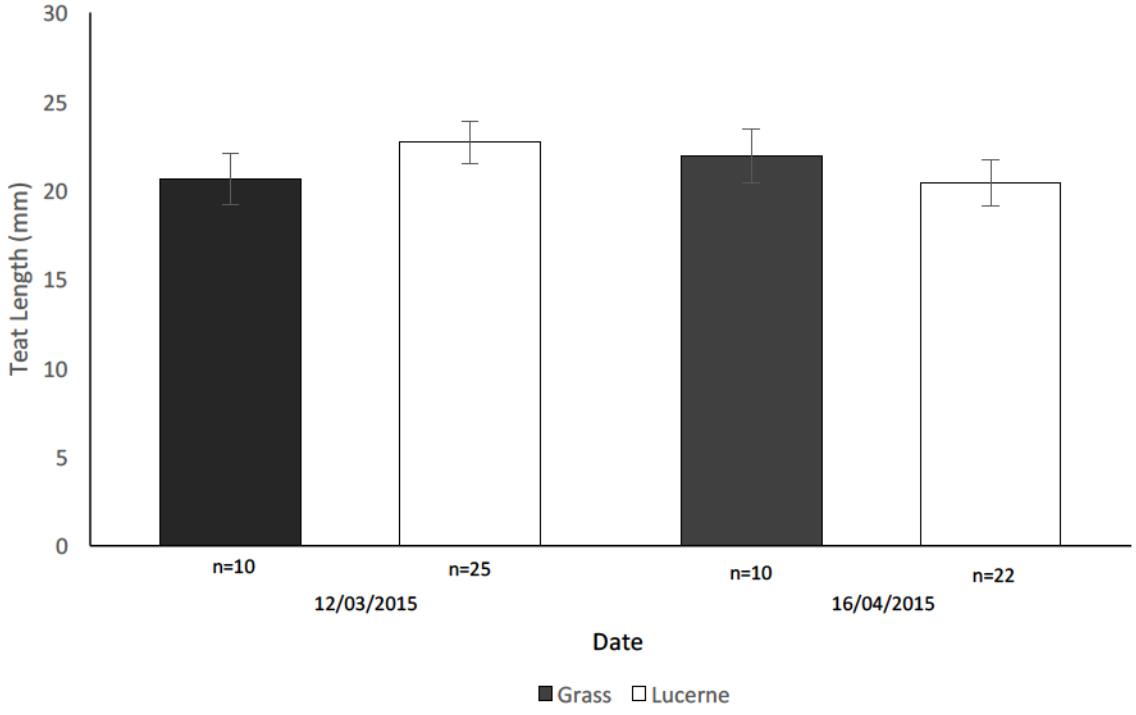

Figure 4.1 Mean teat length[1] of sheep grazing grass or lucerne up to 12 March (left hand bars), then grass only until 16 April (right hand bars). Vertical bars represent ± S.E.M.

[1] – means are calculated from the average of the pair for each sheep

There was a grazing-related effect on teat widths recorded on 12 March (Figure 4.2) with much wider (*P* < 0.001) teats on Lucerne lambs (mean 18.0 ± 1.01 mm, n = 24) than the Grass lambs (mean 13.4 ± 1.77 mm, n = 10). On 16 April, teat widths had slightly increased in Lucerne lambs (0.90 ± 1.09 mm to a mean width of 18.9 ± 1.20 mm, n = 21) whereas there had been a major increase in teat width of Grass lambs (by 5.5 ± 0.54 mm to a mean width of 18.9 ± 2.11 mm, n = 10) so that, at this date, there was no difference in teat width between the two groups of sheep (*P* = 0.964).

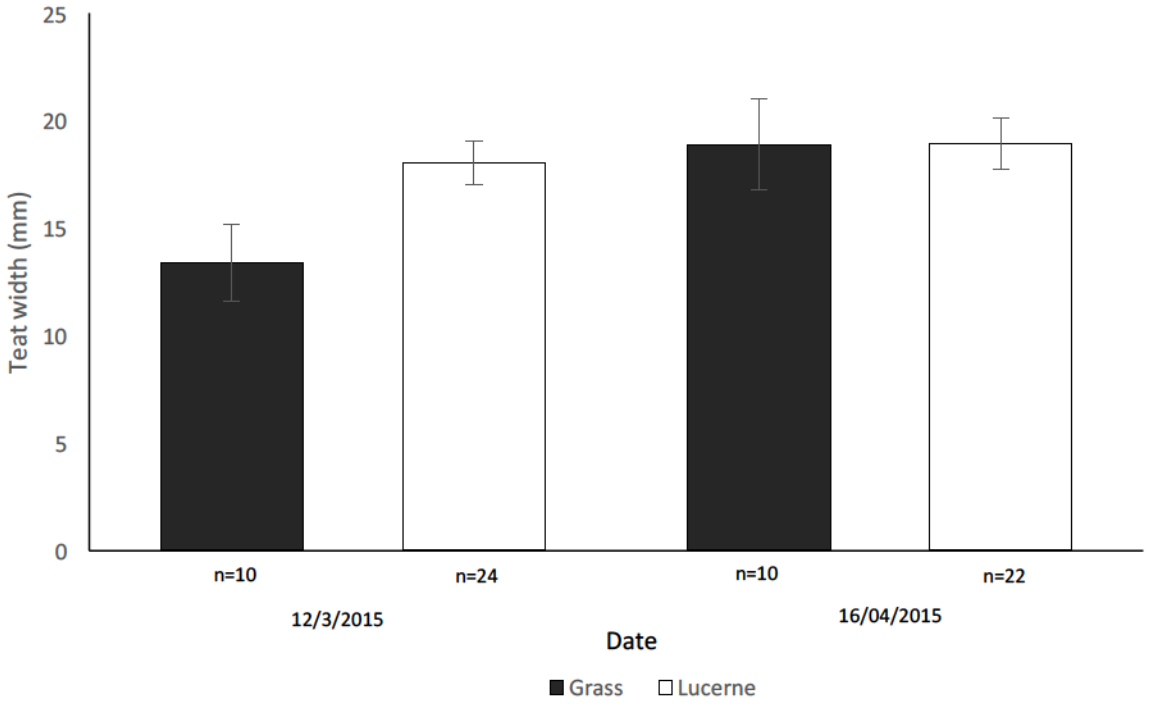

Figure 4.2 Mean teat width[1] of sheep grazing grass or lucerne up to 12 March (left hand bars), then grass only until 16 April (right hand bars). Vertical bars represent ± S.E.M.

[1] – means are calculated from the average of the pair for each sheep

4.1.4 Vulva colour

There was no significant difference ($P = 0.264$) in the vulva colour saturation between grass (57.4 ± 4.88, n = 10) and lucerne grazed ewes (52.8 ± 3.83, n = 25) on 12 March (see Appendix B).

4.1.5 Photographic measurements – comparison with direct (caliper) measurements

There was a significant difference between the measurements taken using the caliper and the slightly smaller measurements obtained from photographs using a ruler for teat width ($P < 0.001$) (see Table 4.2). However, there were no differences between the caliper and photographic measurements for teat length and udder width (see Table 4.2).

	Mean caliper measurement	Mean photo measurement	Difference	*P*-value
Teat width	17.9 ± 0.39	15.3 ± 0.33	2.5 ± 0.39	0.000
Teat length	21.5 ± 0.35	22.1 ± 0.55	0.6 ± 0.42	0.151
Udder width	62.0 ± 1.63	61.6 ± 1.54	0.3 ± 2.00	0.826

Table 4.2 Mean (± s.e.m.) teat width[1], teat length[1] and udder width[1] measurements (all in mm) recorded with calipers compared with values obtained from photographs and the differences (mean ± s.e.m.) between these measurements. Data include all measurements taken on 12 March and 16 April.

[1] – means are calculated from the average of the pair for each sheep

4.2 Experimental Study

4.2.1 Live weight

Mean live weight of the sheep at the beginning of the experiment was 42.8 (± 5.58) kg. Throughout the period of the study there was a general increase in live weight (Table 4.3) but this was not affected by treatment with oestradiol (*P* value = 0.727) (see Table 4.3).

	Live weight day 1 (kg)	**Live weight day 19 (kg)**	**Live weight day 40 (kg)**	**Live weight gain (g/ day)**
Control	41.4 ± 3.61	48.0 ± 3.27	52.5 ± 2.89	284.6 ± 32.15
Low oestradiol	43.8 ± 1.68	50.3 ± 1.27	55.8 ± 1.27	307.7 ± 16.32
High oestradiol	43.1 ± 4.13	50.0 ± 4.28	55.5 ± 4.20	318.0 ± 36.65

Table 4.3 Live weight recorded on 3 occasions in a period of 40 days and live weight gain of sheep (n = 4 per treatment) treated with 0.1 mg oestradiol every 3 days (Low oestradiol), 1.0 mg oestradiol every 3 days (High oestradiol) or not treated (Control).

4.2.2 Mammary development

Mammary development occurred only in the sheep on the high dose of oestradiol (see Appendix B). This was first observed in one animal at 6 days after commencement of treatment with oestradiol, but all the sheep in this group eventually developed udders by the end of the study. For these sheep, the mean mammary gland diameter when it became visually prominent was 50.62 ± 0.65 (n = 4) and was unchanged (P = 0.796) on day 40 (mean diameter 49.92 ± 2.20, n = 4). The mean circumference of the udder when it became visually prominent was 15.50 ± 0.96 (n = 4) and was unchanged (P = 0.103) on day 40 (mean circumference 14.00 ± 1.08, n = 4). There was no visible evidence of mammary development in the Control sheep or the Low oestradiol sheep throughout the experiment.

There was a trend for a positive dose-related effect of oestradiol treatment on mean mammary gland weight on day 41 (Table 4.5) but this was not significant (P = 0.314).

4.2.3 Teat size

Changes in mean teat length are shown in Figure 4.3. There was dose-related effect of oestradiol treatment on teat length ($P < 0.05$) with the sheep that received the high dose of oestradiol having a mean increase in teat length of 3.97 ± 2.21 mm and those in the control and low dose oestradiol groups having slight decreases in mean teat length (reductions of 1.30 ± 2.05 mm and 0.62 ± 5.02 mm, respectively).

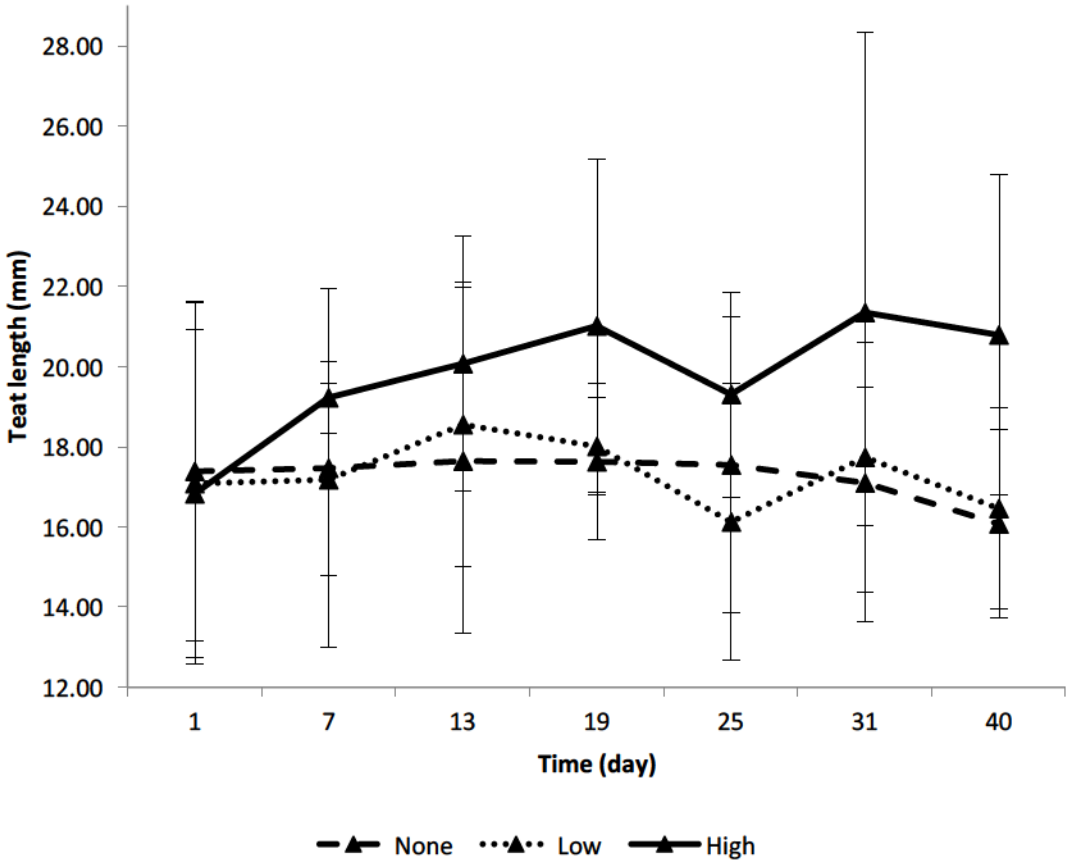

Figure 4.3 **Teat length[1] recorded every 3 days in a period of 40 days of sheep (n = 4 per treatment) treated with 0.1 mg oestradiol every 3 days (Low oestradiol), 1.0 mg oestradiol every 3 days (High oestradiol) or not treated (Control). Vertical bars represent ± s.e.m.**

[1] – means are calculated from the average of the pair for each sheep

Results for teat width are shown in Figure 4.4. There was no effect of oestradiol treatment on mean teat length during the period of the study.

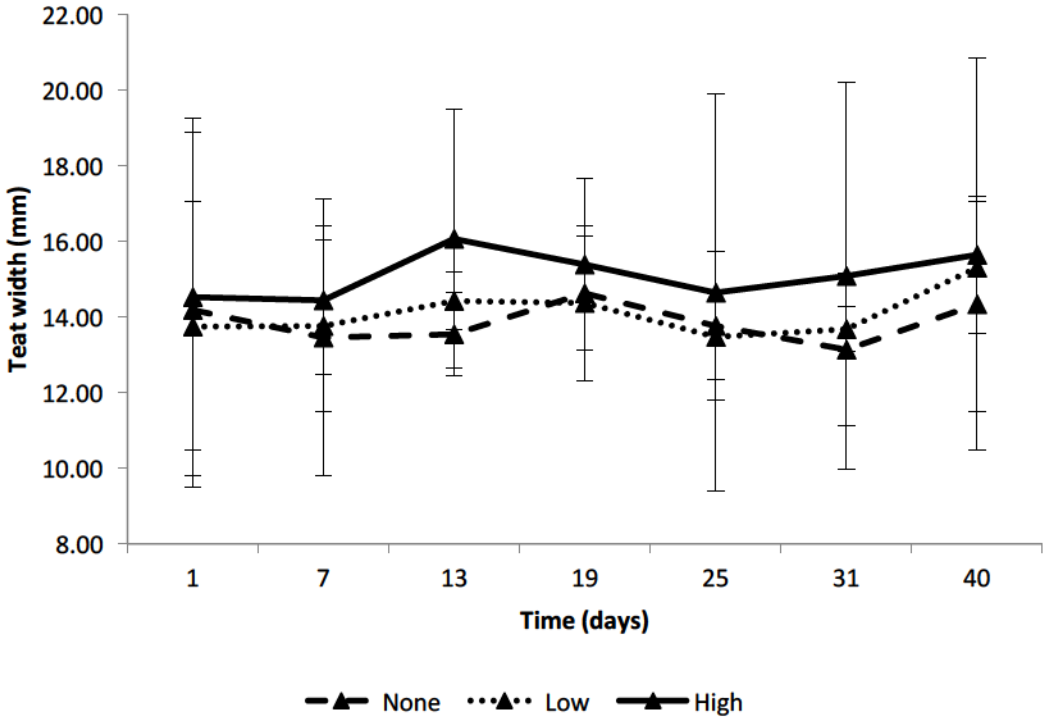

Figure 4.4 Teat width[1] recorded every 3 days in a period of 40 days of sheep (n=4 per treatment) treated with 0.1 mg oestradiol every 3 days (Low oestradiol), 1.0 mg oestradiol every 3 days (High oestradiol) or not treated (Control). Vertical bars represent ± s.e.m.

[1] – means are calculated from the average of the pair for each sheep

4.2.4 Mammary development and teat size determined from photographs

There was a significant difference between the measurements taken using the caliper and the measurements taken using a photograph with a ruler in present view for teat length ($P < 0.001$) across all the measurements taken throughout the duration of the 40-day trial. There was no significant difference between the caliper and photographic measurements taken for teat width ($P = 0.932$) or udder width ($P = 0.179$) (see Table 4.4).

	Mean caliper measurement	Mean photo measurement	Difference	*P*-value
Teat width	14.4 ± 0.21	14.4 ± 0.25	0.0 ± 0.23	0.932
Teat length	18.1 ± 0.27	19.6 ± 0.36	1.4 ± 0.29	0.000
Udder width	52.9 ± 1.25	54.9 ± 1.75	2.0 ± 1.39	0.179

Table 4.4 Mean (± s.e.m.) teat width[1], teat length[1] and udder width[1] measurements (all in mm) when taken using the calipers compared to when taken using a photograph with a ruler in present view and the mean (± s.e.m.) difference between the two measurements. Data includes all measurements throughout the duration of the 40-day trial

[1] – means are calculated from the average of the pair for each sheep

4.2.5 Reproductive tract

Uterus weight

There was a trend for a positive dose-related effect of oestradiol treatment on uterus weight (Table 4.5) but this was not significant (*P* = 0.1).

Ovary weight

There was a trend for a positive dose-related effect of oestradiol treatment on ovary weight (Table 4.5) but this was not significant (*P* = 0.782). There was no dose-related effect on the number of follicles present in the ovaries (*P* = 0.48) (Table 4.6). No corpora lutea were present in any ovaries.

	Control	Low oestradiol	High oestradiol	P-value
Mammary weight (g)	201.3 ± 11.99	234.25 ± 27.79	243.00 ± 13.64	0.314
Uterus weight (g)	28.3 + 1.76	36.3 ± 5.27	44.0 ± 7.33	0.1
Ovary weight (g)	1.44 ± 0.22	1.59 ± 0.07	1.64 ± 0.29	0.782
Vulva redness (°)	168.8 ± 19.97	196.8 ± 2.05	184.2 ± 3.69	0.296
Vulva saturation (%)	53.2 ± 2.45	40.4 ± 3.73	48.0 ± 1.49	0.027

Table 4.5 Post slaughter measurements (mean ± s.e.m.) of mammary weight, uterus weight, ovary weight[1] and vulva colour recorded on day 41 of sheep (n = 4 per treatment) treated with 0.1 mg oestradiol every 3 days (Low oestradiol), 1.0 mg oestradiol every 3 days (High oestradiol) or not treated (Control).

[1] – means are calculated from the total weight of the pair for each sheep

	Control n = 4	Low oestradiol n = 4	High oestradiol n = 4
Follicles > 5mm	5	5	4
Follicles > 8mm	3	1	2

Table 4.6 Number of follicles within diameter size ranges listed here recorded post slaughter on day 41 of the sheep of sheep (n = 4 per treatment) treated with 0.1 mg oestradiol every 3 days (Low oestradiol), 1.0 mg oestradiol every 3 days (High oestradiol) or not treated (Control).

4.2.6 Vulva colour

There was no effect of oestradiol treatment on mean vulva redness during the period of the study (Table 4.5). However, there was an oestradiol dose-related variation in vulva colour saturation (see Table 4.5 and Appendix D).

Chapter 5

Discussion

This study has measured the stimulatory effects of oestrogenic lucerne on the mammary glands and teats of pre-pubertal sheep in New Zealand. It has attempted to relate the oestrogenic effects recorded on-farm to those produced by a precise dose administration of oestradiol to sheep. Also, this study has demonstrated the utility of a photographic procedure for replacing the laborious direct measurement of mammary and teat development but has ruled out the potential of photography to quantify oestrogen-induced colour changes in external genitalia.

Investigation of the effects that phyto-oestrogens have on the reproductive organs of sheep is surrounded in complexity. This is due to the impact that a number of incidental factors that an animal is exposed to could have on the outcome. Incidental factors include plant nutrient composition, simultaneous effect of other hormones and possible involvement of inhibitors and potentiators of oestrogenic activity (Oldfield ***et al.,*** 1966). The on-farm experimental study carried out was an incidental finding on a farm that showed dramatic effects of an oestrogenic crop. The sheep were given ***ab libitum*** access to oestrogenic feed so it was not possible to measure the feed intake of an individual animal. This limits the ability to associate an individual response with level of oestrogenic pasture consumed. An experimental trial was carried out in attempt to quantify the precise amount of oestradiol that must be administered to produce effects of a similar magnitude to those produced by the lucerne crop. This was expected to provide a more accurate assessment of the dosage levels experienced by sheep in the field. It is evident that the sheep used in the experimental trial were much less sensitive to the effects of an exogenous oestrogen than the lambs in the farm study and this lack of responsiveness was probably due to the greater degree of maturity of the oestradiol treated sheep – i.e. they were older.

On average, the lambs (25 – 28 weeks of age) grazing lucerne from the sheep farm trial weighed 45.4 kg therefore were eating 1.82 kg DM /day (based on intake of 4% of body weight). The oestrogenicity of the lucerne crop was analysed to be between 99.8 – 151.41 µg oestradiol equivalent /kg DM therefore the lambs were receiving the equivalent of 181.2 – 274.9 µg oestradiol equivalent /day. The older sheep (42 - 44 weeks of age) were treated with 0, 33.3, 333.3 µg oestradiol /day. Although, the lambs received a considerably lower dose of oestradiol than the older sheep, they were more sensitive and responsive to the oestradiol dose. Older sheep are less responsive due to changes that occur during puberty that influences their sensitivity to exogenous oestrogens. Puberty is defined as

the first time that an animal can sexually reproduce and many researchers use first oestrous as an indicator of puberty. Reproductive organs develop relatively slowly and are functionally inactive in the early life of pre-pubertal lambs (Hafez, 1952). Protection against exogenous oestrogen after puberty occurs could be due to endogenous steroid hormones that are produced during puberty interfering with receptor binding. A range of hormone steroids are produced after the onset of puberty, any of which could be interfering with oestrogen receptors by blocking or inhibiting receptor binding. Pubertal changes making sheep less sensitive to the effects of exogenous oestrogens is supported by entire rams not being affected by oestrogens while castrated males are. Bennetts *et al.* (1946) showed that entire rams are not influenced by 'clover disease' and other studies have shown that there is no oestrogenic effect on puberty, live weight or reproductive traits on entire rams (George and Turnbull, 1966). Castrated males, however, exhibit many effects including change in teat length (Money *et al.,* 1967) and 'clover disease', which includes enlargement of the bulbourethral glands and death (Adams, 1995). Castrated male sheep also have more of a response to oestrogens than entire hoggets. Castrated males showed an increased of 11.4 mm in teat length when dosed oestrogen compared to a 4.8 mm increase observed in entire hoggets (Money *et al.,* 1967), showing that when sheep are not producing their own hormones (castrated, pre-pubertal, ovariectomized) they are more sensitive and responsive to the effects of oestrogens.

More of an effect on teat size was observed in pre-pubertal lambs than pubertal sheep. Lambs grazing oestrogenic lucerne had bigger teats than those grazing grass (4.6 mm wider and 2.1 mm longer). This is in accordance with previous research (Oldfield *et al.,* 1966; Adams, 1995). There was little to no correlation of live weight with teat length and teat width (r^2 =0.00) and live weight corrected data still had a positive effect of oestrogenic pastures. Four weeks after the sheep were removed from the lucerne crop to a rye-grass/white clover pasture, more measurements were taken and there was no difference between the lambs that were previously grazing lucerne and those grazing grass. This is consistent with previous research by Nwannenna *et al.* (1995) who noticed that it took 25 days for teat size to return back to normal after removal from oestrogenic red clover. The sheep in the experimental trial experienced a slight increase in teat length (3.97 ± 2.21 mm) when given high doses of oestradiol and no change in teat width. Teat length and teat width were both moderately correlated with live weight (r^2 = 0.33 and 0.72, respectively) and most of the changes in the study could be explained by live weight differences. Teat growth is allometric and oestrogens cause a stimulatory effect that causes it to grow more than it would with linear growth of an animal. There was daily variation in teat length and width, which is put down to the environmental effect on teat size.

Oestradiol treatment and oestrogenic pastures causes mammary development to occur in both pre-pubertal lambs and pubertal sheep; including mammary gland weight of pubertal sheep. This is in agreement with other studies and the increase in mammary development observed is due to a greater development of glandular tissue and less connective tissue development (Adams, 1977; Valderrabano *et al.,* 1988). Proliferation of mammary epithelial is driven by the sex steroids including oestrogens. Oestrogens exert an effect on the mammary gland and the biological responses are predominantly mediated by receptors, mainly oestrogen receptor (ERα). This oestrogen receptor mediated signalling is essential for ductal development. Development of the mammary gland is minimal and isometric until the onset of puberty, when mammogenesis rapid and allometric (Lamote *et al.,* 2004). There was a moderate correlation between live weight and mammary gland weight of the experimental sheep (R^2 = 0.29) and this can be explained by mammary gland growth being allometric. When data was live-weight corrected there was still a positive dose-related effect of oestradiol.

It is not surprising that the teats and the mammary gland responded to oestradiol at different times. The udder is comprised of two different organs: the glandular tissue (mammary gland) and the mammary skin (teat). There are different cells in the two organs and likely respond to oestrogens differently. In the experimental study, it appears that the glandular tissue responds before the mammary skin to exogenous oestrogen.

There was a positive dose related effect of oestradiol treatment on live-weight in the experimental study. High oestradiol sheep grew 10 g/day faster than low oestradiol sheep and 33 g/day faster than control sheep. Oestrogens have widely been used as growth promoters in sheep as they result in improved growth rates and improved feed utilisation due to a small increase in feed intake. They increase lean tissue deposition (muscle, bone and connective tissue) and decrease subcutaneous and intramuscular fat deposition (Hancock *et al.,* 1991). In the sheep farm study, there was also an oestrogenic effect with ewe lambs grazing oestrogenic lucerne having higher growth rates than ewe lambs grazing ryegrass. Lucerne is a high quality feed source with a high crude protein content and grazing animals experience heightened live weight gain relative to other forages such as grass species, which either don't contain phyto-oestrogen or have a very low concentration. Lambs and sheep are often grazed on lucerne to increase live weight for either slaughter or to flush ewes on prior to mating due to heightened live weight increasing ovulation (Smith *et al.,* 1979; Rattray *et al.,* 1980; Mills *et al.,* 2008).

Endogenous oestrogens cause swelling and increased redness of the vulva and it is reported that coumestans and isoflavones consumption may exert similar effects. When sheep graze oestrogenic

pastures or are treated with oestradiol-17β, the vulva undergoes physical changes such as swelling up and changing in colour. This change may be induced by the increased blood flow that accompanies hypertrophic and hyperplastic enlargement of the reproductive organs (Nwannenna *et al.,* 1995). There was no clear effect of oestradiol treatment or grazing oestrogenic pastures on the saturation and redness of the vulva in both the sheep farm study and the experimental trial. There was a significant effect of oestradiol treatment on vulva saturation, with the vulvas of Low oestradiol treatment sheep being less saturated (vulva saturation values were 53.15% ± 2.45, 40.4% ± 3.73 and 48.0% ± 1.49 for Control sheep, Low oestradiol sheep and High oestradiol sheep, respectively). However, having such a small sample size means that significant values happen by chance and intuitively the significance in vulva saturation is unlikely to have any meaning due to the change being observed in the Low oestradiol group. The discrepancies between these results and other previous studies is due to all of the trials having small sample size therefore changes in the colour of the vulva associated with oestrogen concentration can no be confirmed. Environmental effects such as lightening may also cause difference in vulva red and colour saturation.

Photographic images were taken to see if there was a rapid, easy way for farmers to measure the teat size of sheep. It is expected that there would be good relationship between teat size taken using calipers and teat size taken off a photograph with a ruler in present view. There is error in both methods of measuring and the photograph taken with a relaxed teat and a ruler could be considered more reliable due to the caliper measurement depending on where you touch it and where you squeeze the caliper to. In the sheep farm study there was no significant differences between the two measurements for teat length and udder width (mean differences of 0.61mm ± 0.42 and 0.37mm ± 1.66, respectively). There was a significant difference between the two different measurements for teat width (mean difference of 2.50mm ± 0.39). Regression modelling gave R^2 values of 0.09, 0.41 and 0.22 for teat width, teat length and udder width, respectively. The experimental trial showed that there was no significant difference between the caliper measurement and the photographs with a ruler in present view measurement for teat width and udder width width (mean differences of 0.02 ± 0.23 and 1.99 ± 1.39, respectively). There was a significant difference between the two measurements for teat length (mean difference of 1.43mm ± 0.29. Regression modelling gave R^2 values of 0.14, 0.36 and 0.36 for teat width, teat length and udder width, respectively. For both studies, teat length and udder width gave moderate correlations between the two measurements while teat width was poorly correlation. The two methods of measurements are not highly correlated and is due to angle of the teat in the photograph influencing the photographic measurement. When the photograph had a clear picture of the teat and light was not interfering with it, then there was a good relationship between the two measurements. Taking photographs with a ruler in plain view can

be considered a good enough indicator of the caliper measurement provided that the photos are taken at a good angle.

Oestradiol-17β stimulated uterine growth in treated sheep when compared with their controls. There is a relationship between the live weight of a sheep and its uterus size and regression modelling showed it to be highly correlated with a R^2 value of 0.51. When data was corrected for live weight (expressed as g uterus/kg live weight) there was still a positive dose-related effect meaning that not all of the differences observed in live weight can be attributed to live weight. In the experimental study, High oestradiol sheep had a uterine size of 0.78 g uterus/kg of live weight, Low oestradiol dose sheep had a uterine size of 0.65 g uterus/kg of live weight and Control sheep had a uterine size of 0.51 g uterus/kg. This increase in uterine weight has been accredited to real growth of the uterus rather than water accumulation (Newsome and Kitts, 1980). Uterine growth is in response to the uterus becoming more sensitive to oestrogen stimulation, which increases uterine blood flow causing the uterus to grow, cell proliferation to occur and hyperemia (Reynolds *et al.,* 1998). A study done by Newsome and Kitts (1980) showed that the maximum uterine response is when lambs are given 10 mg of oestradiol per day. Lambs given this dose had uterus weights of 0.6 g uterus/kg of live weight compared to control sheep who had uterus weights of 0.37 g uterus/kg of live weight. This study also looked at the response of uterus in lambs who were treated with 132 µg coumestrol/day (0.132 mg/day) and it showed that although coumestrol-treated ewes received a considerably smaller concentration than the other groups, it still exerted an effect of 0.46 g uterus/kg of live weight. This response was similar to that of lambs who received 2.5 mg/day (2500 µg/day) of oestradiol, showing that phyto-oestrogens exert more of an effect than oestradiol making pre-pubertal lambs more sensitive to them. It is still not known if phyto-oestrogens influence uterine growth in a positive manner or delays puberty as an antagonist.

Previous research indicates that oestrogenic pastures and oestradiol suppress ovarian activity, evident in a reduction in ovarian weight (Newsome and Kitts, 1980; Valderrabano *et al*., 1988; Smith *et al.,* 1990). Grazing oestrogenic pasture also results in sheep developing high numbers of small and medium follicle, however, this abnormal development is accompanied with early atresia (Adams, 1977). Phyto-oestrogens have an inhibitory effect on ovarian function, however, not all phyto-oestrogens have an equal effect on ovarian activity and coumestans are thought to have the biggest influence. Ewes ingesting phyto-oestrogens fail to ovulate and their follicles are histologically abnormal and similar to the follicles of ewes that don't display oestrous. Inhibition of oestrous is due to the ovary failing to secrete endogenous oestrogens, which are what causes sheep to display oestrous. Phyto-oestrogens cause an abnormal relationship between the pituitary and ovaries

resulting in decrease endogenous oestrogen production (Adams *et al.,* 1979; Kelly *et al.,* 1976). This study is in disagreement with previous research. There was no difference observed in follicle numbers then what was expected and no corpus luteum were present in any of the ovaries. Sheep given oestradiol experienced an increase in ovary weight rather than a decrease. Even though there is a weak correlation between live weight and ovary weight (R^2 = 0.08), when ovary weight was corrected for live weight there was no differences in ovary size across the treatments. Detection of the changes in ovarian weights has largely been studied in pre-pubertal lambs; intact adult sheep could be largely protected from the effects of oestradiol so there are not the dramatic changes that are observed in pre-pubertal sheep. However, older intact sheep are not entirely protected evident by the changes observed in ovary weight.

Conclusion

The purpose of this dissertation was to measure the effects that oestrogenic lucerne had on pre-pubertal lambs and to quantify the exact amount of oestradiol that must be administered to produce effects of a similar magnitude to those produced by the lucerne crop.

Results showed that oestrogenic lucerne resulted in mammary gland development and an increase in teat width and teat length of pre-pubertal lambs. Treatment with oestradiol did not achieve effects of the same magnitude as those produced by the lucerne crop due to using older, pubertal sheep. The pubertal sheep in the experimental study were receiving more oestradiol than the pre-pubertal lambs in the sheep farm study (333.3 µg oestradiol /day vs 98.6 – 149.6 µg oestradiol equivalent /day) yet were less sensitive to the oestradiol effect evident by the smaller changes in teat size than the lambs. There were small effects in the pubertal sheep but they were not of the same magnitude as in the pre-pubertal lambs grazing lucerne. The sheep were most likely less responsive to exogenous oestrogen due to oestrogen receptor sensitivity and sensitivity changes with the onset of puberty due to production of their own hormones protecting them against exogenous hormones.

The use of photographs to measure mammary and teat development, can be a good measurement provided that the photo is taken at such an angle that the full teat length and width can easily be distinguished and light doesn't not interfere with mammary and teat boundaries. Photographs are not a useful utility in quantify oestrogen-induced colour changes in external genitalia.

Further research opportunities

Further research is required on the mechanism that make entire sheep less sensitive than pre-pubertal lambs and sheep that aren't producing their own steroids (gonadectomised,

ovariectomized). There is limited literature surrounding oestrogen receptor sensitivity and the effects that exogenous oestrogens have on responsiveness.

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Appendix A

Mammary gland photographs from sheep farm study

Mammary gland development from 12 March (left) and 16 April (right) from a sheep grazing grass.

Mammary gland development from 12 March (left) and 16 April (right) from a sheep grazing lucerne noted as minimal udder development

Mammary gland development from 12 March (left) and 16 April (right) from a sheep grazing lucerne noted as pronounced development.

Appendix B

Vulva photographs from sheep farm study

Vulva colour from 12 March of a sheep grazing grass

Vulva colour from 12 March of a sheep grazing lucerne noted as minimal udder development

Vulva colour from 12 March of a sheep grazing lucerne noted as pronounced udder development

Appendix C

Mammary gland photographs from experimental study

Mammary gland photograph of a High oestradiol sheep (see text) showing the change in mammary gland size from day 1 (left) to day 40 (right)

Mammary gland photograph of a Low oestradiol sheep (see text) showing the change in mammary gland size from day 1 (left) to day 40 (right)

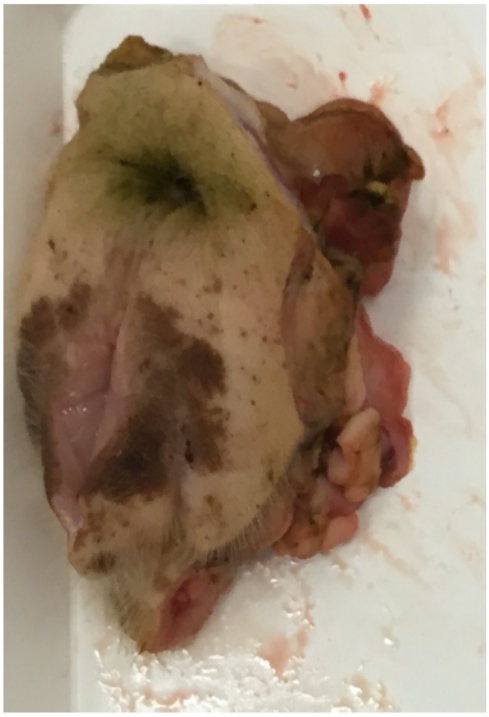
Mammary gland photograph of a Control sheep (see text) showing the change in mammary gland size from day 1 (left) to day 40 (right)

Appendix D

Vulva photographs from experimental study

Vulva photograph typical of a Control sheep (see text).

Vulva photograph typical of a Low oestradiol sheep (see text).



Vulva photograph typical of a High oestradiol sheep (see text)